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MIRNA-125 FAMILY MEMBERS EXERT A SIMILAR ROLE IN THE REGULATION OF HEMATOPOIESIS

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ABSTRACT

MicroRNAs (miRNAs) are crucial for proper functioning of hematopoietic stem and progenitor cells (HSPCs). Members of the miRNA-125 family (consisting of miR-125a, miR-125b1 and miR-125b2) are known to confer a proliferative advantage on cells upon overexpression, to decrease the rate of apoptosis by targeting pro-apoptotic genes and to promote differentiation towards the myeloid lineage in mice. However, many distinct biological effects of the three miR-125 species have been reported as well. In the current study we set out to assess whether the three miRNA-125s that carry identical seed sequences could be functionally different. Our data show that overexpression of each of the three miR-125 family members preserves HSPCs in a primitive state *in vitro*, results in a competitive advantage upon serial transplantation and promotes skewing towards the myeloid lineage. All miR-125 family members decreased the pool of phenotypically defined Lin⁻Sca⁺Kit⁺CD48⁻CD150⁺ long-term hematopoietic stem cells (LT-HSC), while simultaneously increasing the self-renewal activity upon secondary transplantation. The downregulation of miR-125s in HSCs abolishes these effects and impairs long-term contribution to blood cell production. The introduction of a point mutation within the miRNA-125 seed sequence abolishes all mentioned above effects and leads to the restoration of normal hematopoiesis. Our results show that all miR-125 family members are similar in function, they likely operate via seed sequence dependent manner, and induce a highly comparable hematopoietic phenotype.

INTRODUCTION

The limited lifespan of differentiated blood cells requires their constant replenishment by hematopoietic stem cells (HSC). A defined pool of HSCs ensures a daily blood cell production exceeding 10^{12} blood cells (Lansdorp, 1998). These rare and unique cells are able to avoid exhaustion by balancing between self-renewal and differentiation. Stem cell homeostasis is regulated by multiple mechanisms that affect gene expression patterns. One of these mechanisms for fine-tuning gene expression networks is regulated by microRNAs (miRNAs). These are small, non-coding RNAs, that can pair with the 3' UTR (untranslated region) of their target mRNA, leading to its destabilization, resulting in degradation or translational repression (Bartel, 2004). More than 80% of miRNA targets are recognized by the seed sequence, a 5-7 nucleotide, highly conservative sequence, although seed-sequence independent targets are also known. Alternative target recognition sites operate via extensive pairing of the 3' end, or the central 11-12 contiguous nucleotides of the miRNA, with the 3' UTR of the target transcript (Elefant et al., 2011; Engelmann and Spang, 2012). The seed sequence determines the classification of different miRNAs into families. Importantly, a single miRNA can potentially target hundreds of transcripts, generating an extensive network of interactions (Bartel, 2009b).

Previously it has been shown that miRNAs are crucial for the functioning of hematopoietic stem and progenitor cells (HSPCs) (Guo and Scadden, 2010). In the absence of Dicer that cleaves precursor miRNA transcript to mature miRNA (Winter et al., 2009), HSPCs exhibited impaired function and an increased rate of apoptosis. Further studies focused on the identification of miRNAs that are relevant for primitive hematopoietic cells (Gerrits et al., 2012; Guo and Scadden, 2010; O'Connell et al., 2010; Ooi et al., 2010). Members of the miRNA-125 family (consisting of miR-125a, miR-125b1 and miR-125b2) have been shown to be highly expressed in Lineage⁻Sca-1⁺c-Kit⁺ (LSK) hematopoietic cells while their expression decreased upon differentiation (Gerrits et al., 2012; Ooi et al., 2010). The microRNA-125 family members -125a, -125b1 and -125b2 are located on 3 different chromosomes (in the murine genome these are chromosomes 17, 9, and 16, respectively). All family members are most likely expressed in clusters with other miRNAs (Gerrits et al., 2012; Ooi et al., 2010).

Although all miR-125 family members have been shown to provide a proliferative advantage on cells upon their overexpression (Gerrits et al., 2012; Guo and Scadden, 2010; O'Connell et al., 2010), decrease the rate of apoptosis by down-regulating pro-apoptotic genes (Guo and Scadden, 2010; Ooi et al., 2010; Surdziel et al., 2011) and promote differentiation towards the myeloid lineage in mice (Gerrits et al., 2012; Guo and Scadden, 2010; O'Connell et al., 2010), multiple different biological effects of the three miR-125 species have been reported as well. While enhanced self-renewal of HSPCs has been reported (Guo and Scadden, 2010; Ooi et al., 2010), in other studies, including our own, an increase in differentiation was observed (Chaudhuri et al., 2012; Gerrits et al., 2012). Similarly, differences in G-CSF responsiveness have been noticed for miR-125b1 and miR-125b2 in *in vitro* experiments (Surdziel et al., 2011). Also, the extent of differentiation skewing towards the myeloid or lymphoid lineage upon miR-125 family members overexpression has been disputed (Gerrits et al., 2012; Guo and Scadden, 2010; O'Connell et al., 2010). Aside from its effect on normal hematopoiesis, up-regulation of miR-125a and miR-125b expression has been linked to myeloproliferative disorders, acute myeloid leukemia (AML)^{15, 16}, childhood T-cell lymphoblastic lymphoma (Mussolin et al., 2014) while downregulation of miR-125b has been observed in aggressive and indolent chronic lymphocytic leukemias (Tili et al., 2012) in human patients. Therefore members of the miR-125 family may play a role in the etiology of myeloid and lymphoid human malignancies (Bousquet et al., 2008; Ganan-Gomez et al., 2014; Mussolin et al., 2014; Tili et al., 2012).

Although the contribution of miR-125 family members in blood cell production is now well recognized, it remains unclear whether the variable effects described above result from intrinsic differences in effects caused by distinct miR family members, or rather reflect differences of the various experimental models that were used. In the current study we set out to assess whether these miRNAs, that carry identical seed sequences, could be functionally different. To this end we compared all miR-125 family members in various *in vitro* and *in vivo* tests. Our results unequivocally show that all miR-125 family members are similar in function and induce a highly comparable hematopoietic phenotypes that are seed sequence-dependent. Moreover, a single nucleotide mutation of the seed sequence restores hemostasis and documents the seed sequence de-

pendent action of miR-125 family members. Downregulation of miR-125s in HSCs leads to loss of long-term repopulating ability.

METHODS

MICE

Female C57BL/6 (B6, CD45.2) mice were purchased from Harlan and housed under clean conventional conditions. Female C57BL/6.SJL (B6.SJL, CD45.1) mice were bred at the Central Animal Facility of the University of Groningen. All animal experiments were approved by the Groningen University Animal Care Committee.

RETROVIRAL VECTORS

The miR-125a vector was constructed as previously described (Gerrits et al., 2012). MiR-125b1 and miR-125b2 were amplified from genomic DNA from B6 mice (125b1 forward primer: 5'-TTGTGAAGGGGAGAGGTGTAGGG-3', reverse primer: 5'-CGAGTGGCAGCTCCTGGC-3'; miR-125b2 forward primer: 5'-CTCGAGAGCTGTCCGTTTACCTGGAAGAAG-3', reverse primer: 5'-CAATTGGCGCCTATGCAGAACTATGCC-3', Biolegio) and using *XhoI*+*EcoRI* restriction site cloned into MXW pPGK-IRES-eGFP for miR-125b1 and *XhoI*+*MunI* restriction site for miR-125b2 respectively.

The seed sequence mutant T4C miR-125a 5p has been obtained by site-directed mutagenesis using a PCR-TOPO-miR-125a vector with primers containing a point mutation in the 5p arm of the miR-125a stem loop (forward primer: 5'-CTCTGGGTCCCCGAGACCCTTTAACCTG-3', reverse primer: 5'-CAGGTTAAAGGGTCTCGGGGACCCAGAG-3', IDT Integrated DNA Technologies) and Phusion polymerase as suggested by manufacturer (NEB). The resulting PCR product was treated with *DpnI* and used for bacteria transformation. The mutated miR-125a stem loop was cut out from the PCR-TOPO vector using *XhoI*+*EcoRI*, and subcloned into the *XhoI*+*EcoRI* site of the MXW-pPGK-IRES-EGFP vector, which is a murine stem cell virus-based vector with constitutively active Pol II type promoter (Mao and Chen, 2007).

The miR-125a antagomir (later called ZIP) was subcloned from a commercially available vector (SBI) into MXW pPGK- IRES-eGFP vector using XhoI+MfeI restriction sites. The XW pPGK- IRES-eGFP vector was a kind gift from Prof Chen (Stanford University School of Medicine, Stanford, CA).

RETROVIRAL OVEREXPRESSION OF MIR-125A, MIR-125B1, MIR-125B2 OR ZIP

Primary BM cells were isolated from B6.SJL mice 4 days after IP injection of 150 mg/kg 5-fluorouracil (Pharmachemie Haarlem), and cultured in StemSpan (StemCell Technologies) supplemented with 10% FCS, 300 ng/mL recombinant mouse SCF (rmSCF; Peprotech), 20 ng/mL rmIL11 (R&D Systems), 1 ng/mL Flt3 ligand (Amgen), penicillin, and streptomycin. Virus was produced by transfecting Phoenix ecotropic packaging cells with 1-2 µg of pDNA (MXW empty vector, MXW-miR-125a, MXW-miR-125b1, MXW-miR-125b2 or MXW- ZIP) and 3-6 µL of Fugene HD (Roche). Virus-containing supernatant harvested 48 and 72 hours later was used to transduce $4-7.5 \times 10^5$ BM cells per 3.5-cm well. Three independent transductions were performed, per condition, per experiment. Five days after the first transduction, viable (propidium iodide-negative) GFP⁺ cells were FACS-sorted and tested in *in vitro* assays and RNA gene expression studies. Non-sorted cells were tested in an *in vivo* BM transplantation setting. In experiments with the antagomir miR-125a we followed the sorting strategy for stem cells and the transduction protocol described previously (Verovskaya et al., 2013).

QUANTITATIVE PCR VALIDATION

Q-PCR analyses were performed as previously described (Gerrits et al., 2012). To quantify miR-125b1 and miR-125b2 abundance, cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription Kit with specific TaqMan probes to discriminate between precursor-miR-125b1 and precursor-miR-125b2 transcripts (Applied Biosystems).

CAFC ASSAYS

The cobblestone-area forming cell (CAFC) assay was performed as previously described (Ploemacher et al., 1991; Szilvassy et al., 1996; van Os et al., 2008b).

PRIMARY BONE MARROW TRANSPLANTATION

Bone marrow transplantations were performed as previously described (Gerrits et al., 2012). As a source of donor cells B6.SJL mice were used and lethally irradiated (9 Gy) B6 mice served as recipients in both transplantation experiments. In the first experiment mice were transplanted with 10^7 and in the 2nd 7.5×10^6 whole bone marrow cells. In experiments with the ZIP sorted LT-HSC (Lin⁻Sca-1⁺c-Kit⁺CD48⁻CD150⁺), transduced, non-resorted for GFP⁺ cells (3.5×10^4) were transplanted into lethally (9 Gy) irradiated B6 mice, simultaneously with $5-10 \times 10^5$ radioprotective B6 cells. Gene transfer efficiencies were determined at 3 to 5 days post-transduction in an aliquot of cells left in the culture, that were further expanded and FACS-sorted to test overexpression levels.

SECONDARY BONE MARROW TRANSPLANTATION

BM cells from primary miR-125a, miR-125b1 and miR-125b2 and empty vector mice were used for secondary transplantations. Transplanted cells were competed with freshly isolated wild type CD45.2⁺ BM cells in lethally irradiated recipients (CD45.2) at 4:1 (test to freshly isolated) ratios. For each of the conditions 9 mice (3 individual primary mice x 3 secondary recipients/mouse) received transplants with a total of 5×10^6 cells each. Blood analyses were performed every 4-6-weeks post-transplantation.

RESULTS

ECTOPIC EXPRESSION OF MIR-125A, MIR-125B1 AND MIR-125B2 RETAINS HSPCS IN A PRIMITIVE STATE

To overexpress all three members of the miR-125 family in murine hematopoietic cells we have cloned miR-125a, -b1 and -b2 into retroviral vectors in their

natural genomic context (as 250-500 base pair long genomic sequence, Fig. 1A and B). Bone marrow cells of 5-FU treated mice were transduced with the various vectors. Transduced GFP⁺ cells were used to initiate *in vitro* assays and to analyze the overexpression level by quantitative PCR with primers recognizing mature miRNA sequences (Fig. 1C). To discriminate between identical mature miR-125b1 and miR-125b2, we confirmed their respective overexpression using primers recognizing the precursor form of miRNA-125b1 and miR-125b2 (data not shown). Levels of miR-125a, miR-125b1 and miR-125b2 were markedly increased (140-, 200-, 210-fold, respectively) compared to the empty vector control (all differences compared to the empty vector were statistically significant).

GFP⁺ cells were used to initiate cobblestone area forming cell assays (CAFC). In this assay early appearing cobblestones (day 7) are considered to represent differentiated cells and late appearing cobblestones (day 35 and later) are proportional to the number of more immature or primitive cells (Ploemacher et al., 1991; Purton and Scadden, 2007; Szilvassy et al., 1996; van Os et al., 2008b). No differences between the control and miRNAs-overexpressing cells were observed for day-7 CAFC activity. However, at day 35, a striking ~100-fold increase in the frequency of CAFCs was observed in all conditions where miRs were overexpressed. No statistically significant differences were found between the three studied miRNAs (Fig.1D). Furthermore, all miR-125 family members similarly prolonged the CAFC activity until day-60, which is not observed for non-manipulated cells.

Taken together, BM cells overexpressing individual members of the miR-125 family showed strongly increased frequency of HSPCs in *in vitro* assay. This effect was similar between all family members. These results suggest that ectopic expression of miR-125 family members retains cells in a more immature stage.

OVEREXPRESSION OF MIR-125 FAMILY MEMBERS IN HSPCS CONFERS AN IN VIVO PROLIFERATIVE ADVANTAGE AND SPORADICALLY LEADS TO MYELOPROLIFERATIVE DISORDER

Parallel with *in vitro* assays, miRNA overexpressing cells were transplanted into lethally irradiated recipients to assay their long-term competitive repopulation

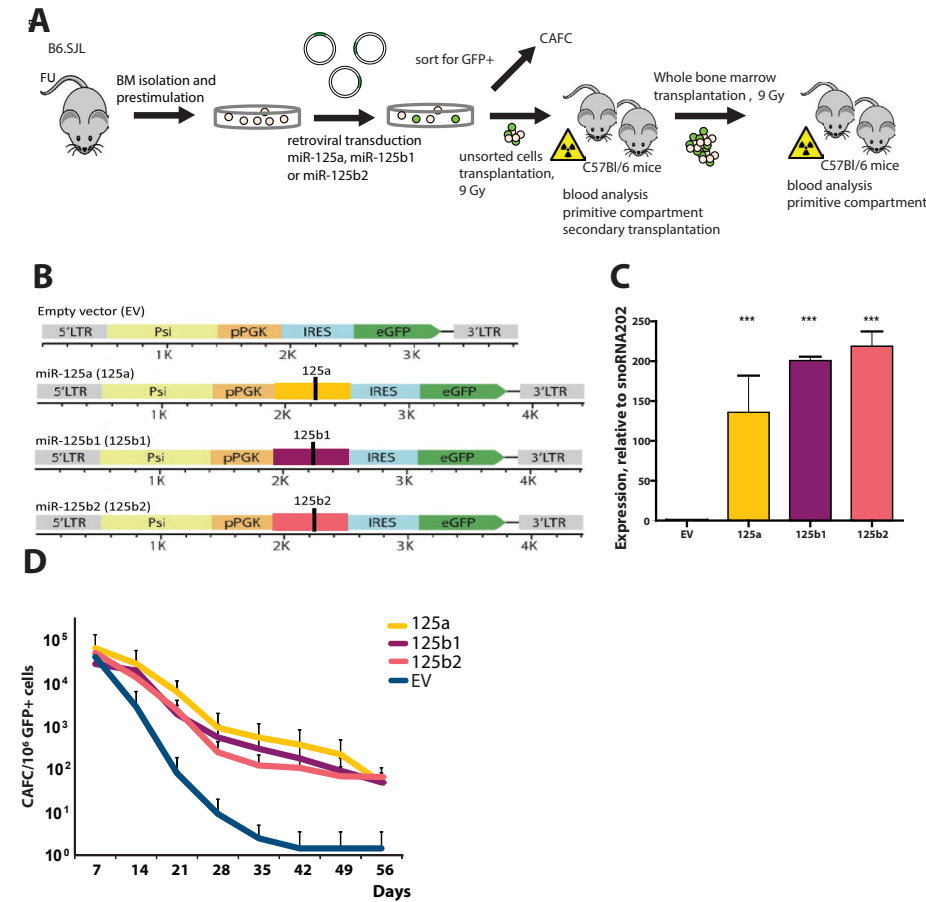


Figure 1. Overexpression of miR-125 family members inhibits differentiation of HSPCs. A) Experimental set up. B) Schematic representation of retroviral vectors used to overexpress miR-125 family members. C) Quantitative RT-PCR results showing the expression levels of miR-125a, miR-125b1 and miR-125b2 relative to the endogenous control snoRNA202, measured in post 5-FU GFP⁺ bone marrow cells. Shown is the mean +SEM. D) CAFC data showing the frequency of HSPCs (in 3 independent experiments). The differences between groups were evaluated by unpaired t-test *P<.01; **P<.01; ***P<.0001.

potential *in vivo* (Fig. 1A). At 10 weeks post transplantation, blood cell counts revealed a moderate decrease in red blood cells (RBC) numbers in a proportion of the mice (in the case of miR-125a and 125b2 overexpression) and a strong increase in the number of white blood cells (WBC) in essentially all mice transplanted with cells overexpressing any of the miR-125 family members (Fig. 2A).

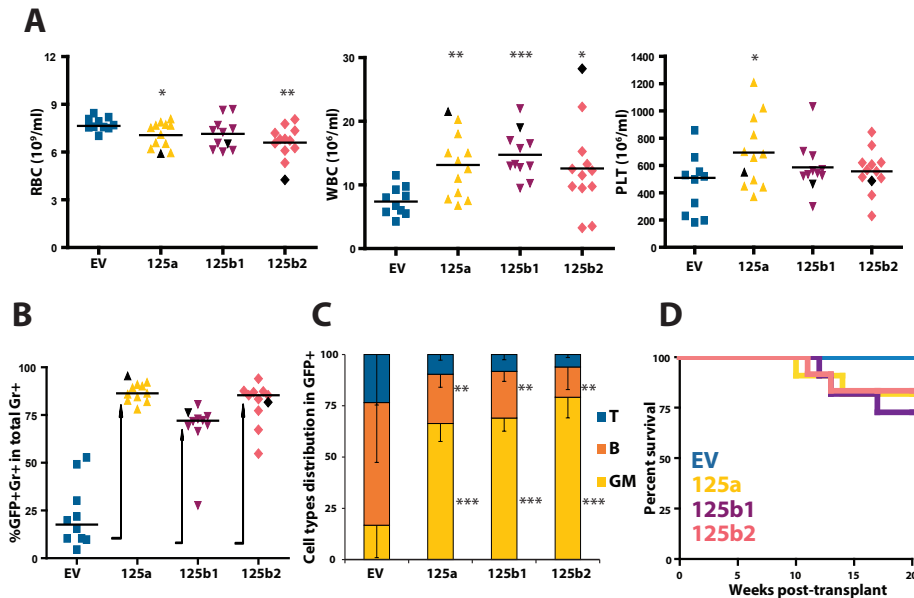


Figure 2. Sustained overexpression of miR-125 family members perturbs hematopoiesis. A) RBC, WBC and PLT 10 weeks after transplantation (n=8-12 mice/group). The differences between groups were evaluated by unpaired t-test *P<.01; **P<0.01; ***P<0.0001. B) Chimerism levels were measured at 10 weeks after transplantation by analyzing GFP+ cells in the donor fraction (n=8-12 mice/group). The arrows indicate the growth of chimerism during first 10 weeks post transplantation. C) Cell types distribution in GFP+ cells in the blood as assessed by FACS (n=8-12 mice/group). Shown is the mean-SD. D) Survival of mice (n=8-12 mice/group). WBC indicate white blood cells; RBC, red blood cells; PLT, platelets; GM; granulocytes/macrophages; B, B lymphocytes; T, T lymphocytes. Closed black symbols indicate moribund miR-125 mice.

Platelet counts were increased in mice reconstituted with cells overexpressing miR-125a (Fig. 2A). Chimerism levels were determined by quantifying the percentage of GFP+ granulocytes in the total fraction of granulocytes (defined as Gr-1+). While within a period of 10 weeks the chimerism levels of the control mice remained stable at ~15%, chimerism in mice transplanted with cells overexpressing miR-125a, -b1, or b2 increased from 8% to 90%, 4.5% to 75% and 5% to 90%, respectively. This suggests that ectopic expression of any miR-125 family member provides a strong competitive advantage over non-transduced cells (Fig. 2B). FACS analysis revealed that cells overexpressing miR-125a, -b1 or -b2 preferentially differentiate into the myeloid lineage (granulocytes and macrophages, defined as Gr-1+ and/or Mac-1+) at the expense of B- lymphocytes (defined as B220+) compared to the control (Fig. 2C). Whereas the per-

centage of GFP+ T cells (CD3+) was decreased, the absolute numbers of GFP+ T cells produced by HSPCs overexpressing miR-125 family members were increased compared to the control (Fig. S1A, B and C). A similar trend was observed for B cells and granulocytes; a pattern which is consistent with previous studies (Gerrits et al., 2012). In one out of two independent transplantation experiments 7 out of 42 mice developed myeloproliferative neoplasms (MPNs), leading to lethality between 10 and 16 weeks post transplantation (2 mice – miR-125a, 3 mice -miR-125b1, 2 mice -miR-125b2) (Fig. 2D). In the second experiment we observed similarly high chimerism levels and mainly myeloid cells in GFP+ compartment, but no lethality (up to 28 weeks post transplantation). Compared to our previous study we have observed a somewhat higher mortality rate in our *in vivo* experiments. As the transductions efficiencies in these experiments were modest (8%, 4.5% and 5% for miR-125a, -b1 and -b2, respectively), most cells will not have integrated more than a single copy vector (Supp. Fig. 2). Therefore we do not consider that insertional mutagenesis contributed to myeloproliferative disease.

To obtain more insight into the type of hematologic malignancy that developed in mice transplanted with miR-125 overexpressing cells, we performed hematological analysis on 6 moribund mice (in one case pathology was not possible). We observed increased WBC counts (Fig. 2A and 3C), splenomegaly (Fig. 3C), pale bones, increased bone marrow cellularity (Fig. 3B), and a large number of blast-like cells in the bone marrow (data not shown). These are similar features as we have reported in our previous paper (Gerrits et al., 2012). Importantly, these phenotypes were very similar for all miR-125 family members and resembled characteristics of previously observed MPNs (Gerrits et al., 2012). Detailed FACS analysis of PB, BM and spleens revealed the dominance of GFP+ cells of myeloid origin (Gr-1+ and/or Mac-1+) (Fig. 3D) and infiltration of those cells in liver and lungs (data not shown).

To summarize, data demonstrate that upon transplantation in mice, stem and progenitor cells in which miR-125 family members are overexpressed, show a competitive advantage, are myeloid skewed and induce a MPN-like phenotype. Although almost all mice transplanted with miR-125a, -b1 and -b2 overexpressing cells display features of MPNs, only 15% of the cases were lethal.

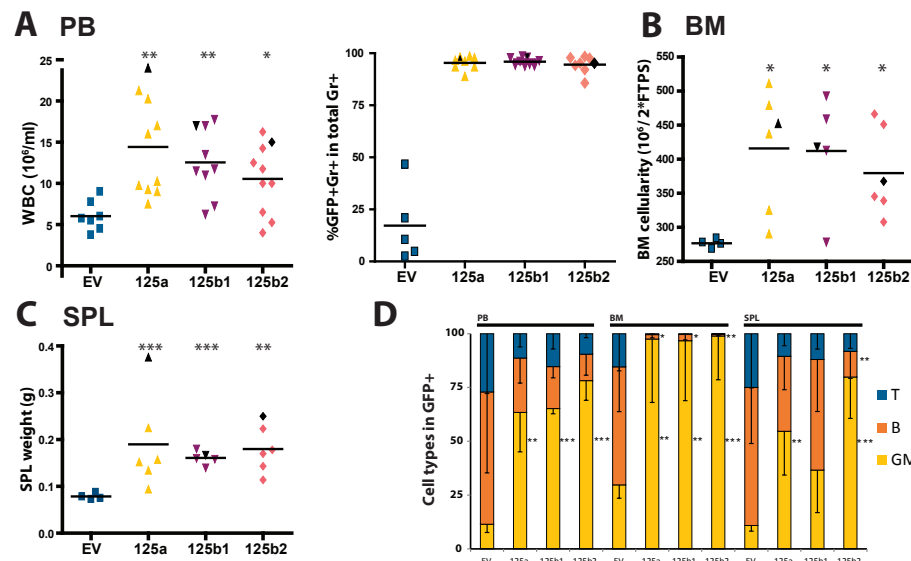


Figure 3. Overexpression of miR-125 family members causes splenomegaly and promotes myeloid differentiation. A) WBC, B) the chimerism levels in the blood and bone marrow cellularity (representing 2 femurs, tibias, pelvic bones, sternum and the spine), C) spleen weight 16 weeks upon transplantation in primary recipients (n=8-12 mice/group). D) Cell types distribution in GFP⁺ in the blood as assessed by FACS (n=8-12 mice/group). Shown is the mean-SD. The differences between groups were evaluated by unpaired t-test *P<.01; **P<.01; ***P<.0001. WBC indicate white blood cells; RBC, red blood cells; PLT, platelets; GM; granulocytes/macrophages; B, B lymphocytes; T, T lymphocytes. Closed black symbols indicate moribund miR-125 mice.

THE COMPETITIVE ADVANTAGE PROVIDED BY MIR-125S IS STABLE OVER TIME, BUT COINCIDES WITH LOSS OF PHENOTYPICALLY DEFINED HSCS

To quantify the number of HSPCs in the BM of healthy miR-125 overexpressing mice we performed immunophenotypic analyses of the primitive BM compartment. We observed a reduced frequency and absolute number of GFP⁺ progenitors (defined as Lin⁻Sca-1⁺c-Kit⁺, LSK; Fig. 4C) and long-term stem cells (LT-HSC, defined as Lin⁻Sca-1⁺c-Kit⁺CD48⁻CD150⁺ cells, Fig. 4B), in the bone marrow of miR-125a, miR-125b1 and miR-125b2 overexpressing mice compared to their control counterparts, 4 months post transplantation (Fig. 4A and B). At the same time we noticed an increased frequency of Lin⁻Sca-1^{mid}c-Kit^{mid}

cells (Fig. 4 A). When calculating the total number of LSK or LT-HSC cells we have taken into consideration the increased bone marrow cellularity as well as higher chimerism levels in miR-125 mice. Phenotypically defined LT-HSC were also absent from spleens or peripheral blood in mice transplanted with cells overexpressing miR-125 family members (data not shown).

To functionally determine the presence of HSCs in bone marrow of primary recipients of miR-125 overexpressing cells, we performed secondary BM transplantations. We assessed the *in vivo* repopulating ability of GFP⁺ cells isolated from the BM of primary recipients compared to freshly isolated BM cells in a competitive transplantation setting.

We observed clear differences in chimerism levels among miR-reconstituted and control mice, 10 weeks and 24 weeks upon transplantation. Three cases of MPNs developed, resembling the features we have described in our primary recipients (Fig. 4E) (Gerrits et al., 2012). Due to the unexpectedly high engraftment levels upon miRNA-125 overexpression in secondary transplanted mice an exact LT-HSC frequency estimate was not possible. Nevertheless, the high chimerism levels in Gr-1⁺ cells at later time points (up to 24 weeks post transplantation) indicates presence of functional LT-HSCs in the bone marrow of mice transplanted with miR-125 family members.

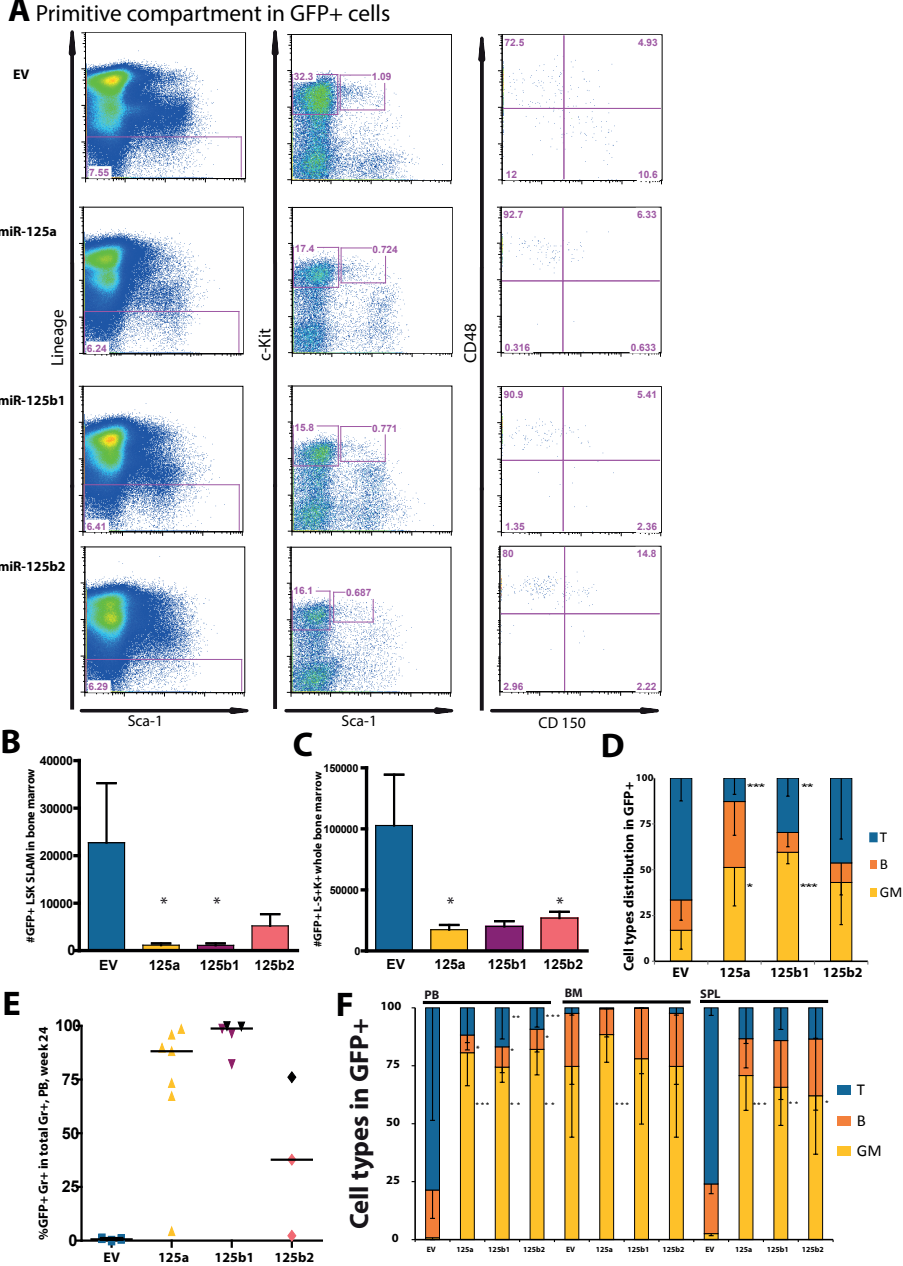
Collectively, our data illustrate that miR-125a, miR-125b1 and miR-125b2 affect hematopoiesis similarly. All three miRNAs conferred a competitive advantage to long-term engrafting hematopoietic cells, but these cells cannot be identified by the expression of commonly used cell surface markers for LT-HSCs.

THE ROLE OF THE MIR-125 SEED SEQUENCE

To better understand the molecular mechanism by which miRNA-125 family members are able to modulate hematopoiesis we tested to what extent the observed phenotype was seed sequence dependent. To address this question, we determined whether a single nucleotide mutation within the seed sequence would affect the miRNA-125 induced phenotype *in vitro* and *in vivo*. Since our previous experiments showed no essential differences between the various miR-125 family members, we performed these studies with miR-125a only. We introduced a T4C mutation in the seed sequence of miR-125a (i.e. the 4th

nucleotide within the seed sequence has been mutated from T to C, Fig. 5A). Next, we transduced highly purified LT-HSCs with a vector carrying mutated T4C-miR-125a 5p and compared this with LT-HSC expressing wild type miR-125a. Overexpression and processing of the mutant T4C miR-125a 5p and miR-125a in LT-HSC were confirmed by quantitative PCR (Fig. 5 F). When we analyzed blood 16 weeks post transplantation cells overexpressing mutant miR-125a did not show clear competitive advantage that was seen with wild type miR-125a overexpressing LT-HSCs (Fig. 5 D). Furthermore, donor cells clearly showed skewing towards the myeloid lineage in mice transplanted with LT-HSCs overexpressing wild type miR-125a, and a compromised frequency of B lymphocytes, which was absent in mice transplanted with cells overexpressing either mutant T4C miR-125a 5p or the empty vector control (Fig. 5E). We also analyzed the effect of depletion of miR-125s in LT-HSCs by transducing highly purified LT-HSCs with a vector carrying an antagomir for miR-125a (Fig. 5A). In transduced cells we confirmed decreased levels of the mature form of miR-125a, as measured by qPCR (Fig. 5B). Since mature miR-125s share over 50% sequence similarity we also have tested the endogenous expression level of miR-125b. This miRNA was below the detection level (data not shown). As expected, cells overexpressing miR-125a clearly showed a proliferative advantage over non-transduced cells. Conversely, cells in which mir-125 was downregulated or mutated hardly contributed to long-term engraftment (Fig. 5C). Observed chimerism levels were low in control mice, however mice transplanted with ZIP miR-125a 5p transduced LT-HSCs were given 17 times higher number of cells compared to controls (8500 transduced GFP⁺ LT-HSC versus 500 GFP⁺ LT-HSC). Moreover 2 million of fresh bone marrow competitor cells were used,

Figure 4. Influence of ectopic miR-125 family members expression on the primitive compartment. A) Representative FACS plots of the GFP⁺ BM compartment 16 weeks post primary transplantations. B) Quantification of the total cell number of LSK SLAM (Lin⁻Sca⁺Kit⁺CD48⁻CD150⁺) and C) LSK (Lin⁻Sca⁺Kit⁺) cells in total bone marrow at 16 weeks after transplantations. D) Cell type distribution in blood of empty vector or miR-125 family members upon secondary transplantation tested by FACS 16 weeks post transplantation (n=3-7 mice/group). E) Chimerism levels 24 weeks upon secondary transplantation, Gr⁺ indicates granulocytes. F) Cell types distribution in GFP⁺ in the blood, bone marrow and spleen of empty vector and miR-125 family members 24 weeks upon secondary transplantation assessed by FACS. Shown is the mean-SEM (n=3-7 mice/group). WBC indicate white blood



cells; RBC, red blood cells; PLT, platelets; GM; granulocytes macrophages; B, B lymphocytes; T, T lymphocytes. Analysis were performed using FlowJo (TreeStar), followed by the quantification of the number of LSK SLAM (n=3-7 mice/group). Differences between groups were evaluated by unpaired t-test *P<.01; **P<.01; ***P<.0001.

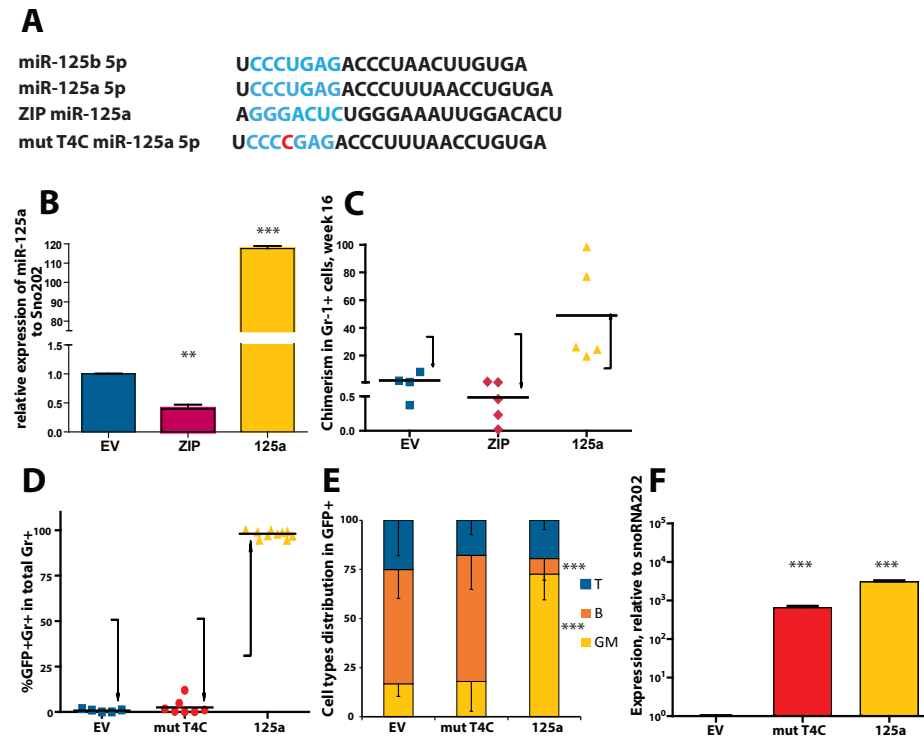


Figure 5. Downregulation of miR-125a abolishes competitive advantage, a single nucleotide mutation within the seed sequence restores normal hematopoiesis. A) The mature sequence of miR-125b 5p, miR-125a 5p and the sequence of ZIP-miR-125a. The seed sequence of miR-125a 5p (depicted in blue) has been mutated T4C (marked in red). B) quantitative RT-PCR results showing the expression levels of miR-125a 5p, in samples in which miR-125a or ZIP-miR-125a 5p is overexpressed, relative to the endogenous control snoRNA202, measured in GFP⁺ cells, sorted 4 days after transduction of LT-HSCs. Mir-125b 5p expression level was below the detection level. Shown is the mean+SEM. C) Chimerism levels were measured at 16 weeks after transplantation by analyzing GFP⁺Gr-1⁺ cells in total Gr-1⁺ fraction (n=4-5 mice/group). The arrows indicate changes in chimerism during first 16 weeks post transplantation. D) Chimerism levels in miR-125a, mutT4C miR-125a 5p and controls were measured at 16 weeks after transplantation by analyzing GFP⁺ cells in total granulocytes (n=4-11 mice/group). The arrows indicate changes of chimerism during first 16 weeks post transplantation. E) Cell-type distribution in the blood as assessed by FACS (n=4-11 mice/group). Shown is the mean+SD. GM indicates; granulocytes/macrophages; B, B lymphocytes; T, T lymphocytes. F) Quantitative RT-PCR results showing the expression levels of miR-125a 5p, mutT4C miR-125a 5p relative to the endogenous control snoRNA202. Shown is the mean+SEM.

that also confer to low contribution of control cells to blood cell production. Nevertheless 3 out of 4 control mice were positive whereas only 2 out of 5 ZIP miR-125a 5p transplanted mice were positive (Fig. 5C).

Taken together our data clearly demonstrate, that miR-125 is indispensable for hematopoiesis. The downregulation of miR-125s leads to the loss of competitive advantage of LT-HSCs. The phenotype induced by miR-125 family members depends on the seed sequence. Even a single nucleotide change within the 7 nucleotide-long seed sequence is sufficient to abolish the aberrant effects in hematopoiesis caused by wild type miR-125a overexpression.

DISCUSSION

In this report we compared the regulatory role of all three miR-125 family members in the biology of HSPCs. All miRNAs that belong to this family induced a striking increase in the number of late appearing CAFCs *in vitro*, and remarkably extended their activity. Furthermore, transplantation experiments demonstrated a very significant competitive advantage of cells transduced with each of the miRNA-125 family members and promoted myeloid differentiation at the expense of the lymphoid lineage. Occasionally, deficiency in the erythroid differentiation and excessive skewing towards the myeloid lineage preceded the development of lethal MPNs. These data are in line with an earlier study in which miR-125b induced MPNs in a dose dependent way (O'Connell et al., 2010).

Interestingly, we were not able to identify phenotypically defined LT-HSCs in the bone marrow of recipient mice, although unfractionated bone marrow from these mice had a strong competitive advantage (and must have contained functional stem cells) in serial transplantations. This suggests that increased levels of miRNA-125a, 125b1 or b2, all lead to enhanced self-renewal. Increased self-renewal of cells overexpressing miR-125b or miR-125a, respectively, has been reported earlier (Guo et al., 2010; Ooi et al., 2010) The observed loss of phenotypically defined LT-HSCs might result from the aberrant expression of the stem cell markers Sca-1 and/or c-Kit, which prevented their identification using FACS analysis.

The decreased frequency of phenotypically, but not functionally, defined LT-HSCs may also partially explain the discrepancies in the literature. Our functional data originating from secondary transplantation studies of cells overexpressing miR-125a, miR-125b1 or miR-125b2 do not support the concept of stem cell exhaustion, which one would expect from the FACS profiles of the BM compartment shown in Figure 4A.

Moreover, inconsistencies that have been reported in the literature regarding the effect of miR-125 family members on hematopoiesis may originate from the genomic context of each miRNA. In our previous paper (Gerrits et al., 2012) we have shown LT-HSC exhaustion upon secondary transplantation when the entire genomic region containing the miR-99b-let7e-125a cluster was overexpressed. It is known that the miRNA-let7 family is involved in the regulation of differentiation *via* a Lin28b-let7e feedback loop (Yuan et al., 2012; Zhong et al., 2010). The expression level of Lin28b is inversely correlated with the expression of mature let-7 family members. High expression level of Lin28b is observed in early stages of embryonic stem cells differentiation and hematopoiesis, and decreases during differentiation, whereas an opposite expression pattern is apparent for let7 miRNAs (Yuan et al., 2012; Zhong et al., 2010). The previously observed effect of ectopic expression of the miRNA cluster 99b-let7e-125a may give an initial competitive advantage to HSPCs but lead to their differentiation and exhaustion upon secondary transplantation, reflecting the opposite effects induced by miR-125a and let7e (Gerrits et al., 2012).

Collectively, our data reinforce the important role of all three miR-125 family members in the regulation of hematopoiesis. We show that all three miRs confer very similar phenotypes when overexpressed, and our studies highlight the essential role of the CCCUGAG seed sequence. It is interesting that we did not detect meaningful biological differences between miR-125a, b1 and b2 during hematopoiesis. The effects of downregulation of mir-125 highlight the loss of competitive advantage of LT-HSCs. Although all three family members appear to exert similar effects, they are located on different chromosomes, and therefore their normal mode of regulation might differ. It is possible that the endogenous expression and the stability of the three mature miRNAs transcripts may be distinct in different cell types, yet control similar self-renewing and differentiation pathways (Ganan-Gomez et al., 2014; Surdzil et al., 2011; Zhou et al., 2009).

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AUTHORSHIP

Contribution: E.E.W, M.A.W, L.V.B. and G.d.H. designed research; E.E.W., M.A.W., M.J.C.B., E.W., M.R., A.A. performed research; E.E.W., M.A.W. and L.V.B. analyzed and interpreted data; and E.E.W., M.A.W., L.V.B. and G.d.H. wrote the manuscript.

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SUPPLEMENTAL FIGURES

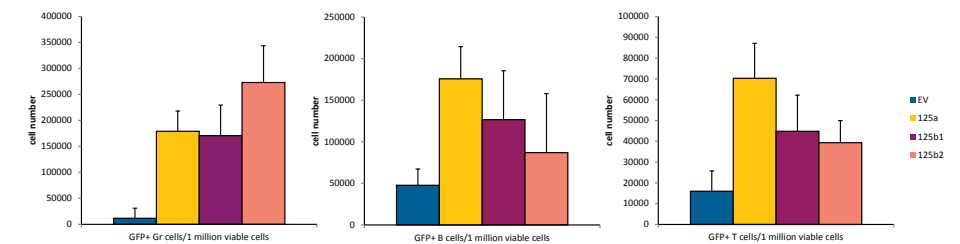


Figure S1. The absolute cell numbers produced by GFP+ cells. A) Total number of Gr-1 cells (defined as Gr-1⁺), B) total number of B cells (defined as B220⁺) and C) total number of T cells (defined as CD3⁺) produced by 1 million viable cells, 10 weeks post transplantation.

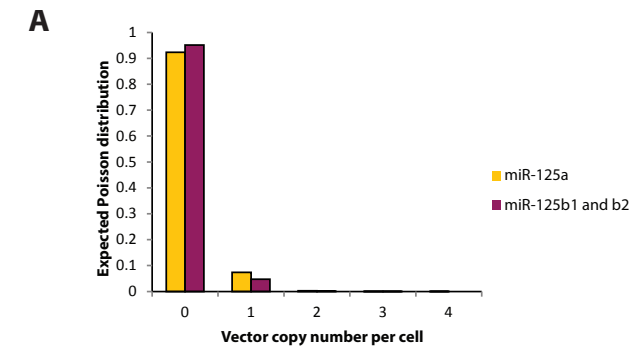


Figure S2 The expected Poisson distribution of vector copy numbers in GFP+ HSPCs. Shown is the distribution of the vector copy number per cell in post 5-FU bone marrow cells in the first experiment.